UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
CHEMICAL SAFETY AND
POLLUTION PREVENTION

May 3, 2017

To: [redacted]

Through: [redacted]

From: [redacted]

Through: [redacted]

[redacted]

RE: Review of Sensitization Data for the PMN substance, P-16-0510

Conclusion: Based on the available data, EPA concluded that P-16-0510 is unlikely to be a sensitizer.

Background: At the Structure Activity Team (SAT) meeting on August 19, 2016, the SAT Chair and SAT Health Assessor determined that P-16-0510 was "inconclusive" for sensitization, because EPA did not consider the results of the two *in vitro* studies submitted with the PMN substance to be sufficient to make a regulatory decision regarding sensitization at that time. The concerns associated with the PMN substance regarding sensitization, as well as other concerns associated with the PMN substance, were communicated to the submitter in an action letter dated March 22, 2017.

On March 17, 2017, the submitter provided full study reports for two additional sensitization studies. The submitter requested that EPA reconsider the initial characterization of the PMN substance as a potential sensitizer. This memo includes the outcome of EPA's review of all submitted sensitization studies, and provides the regulatory decision on P-16-0510 regarding sensitization.

Sensitization Study Reviews and EPA Decisions

In the initial submission, the submitter provided full study reports for two *in vitro* sensitization assays: the Direct Peptide Reactivity Assay (DPRA, OECD TG 442C) and the KeratinoSens™ assay (OECD TG 442D). The report conclusions indicated negative prediction for skin sensitization for both studies. EPA evaluated the studies and agreed with their quality and reliability, but questioned the sufficiency of the data to draw regulatory conclusions with regard to sensitization at that time.

On March 17, 2017, the submitter provided full study reports for two additional studies: the *in vitro* Human Cell Line Activation Test (h-CLAT, OECD TG 442E) and an *in vivo* human sensitization assay (the Human Repeated Insult Patch Test [HRIPT, modified Shelanski – Shelanski method]). The report conclusions indicated negative prediction for skin sensitization for both studies. For the current evaluation, EPA reviewed the results of the newly submitted studies (h-CLAT and HRIPT) and also re-evaluated the initially submitted studies (DPRA and KeratinoSens™ assay). Overall, EPA agreed with the quality, reliability and conclusions of all the studies; summaries of each study are provided in Appendix A.

Based on this comprehensive review, EPA concludes that the combined studies provide sufficient information to conclude that the PMN substance (P-16-0510) is not likely to be a sensitizer. However, additional hazard concerns other than sensitization remain based on analogy to acrylamide, as described in the SAT report and in the letter to the submitter dated March 22, 2017.

References

Carathers, MR. Human Cell Line Activation Test (h-CLAT). MB Research Labs. Research Project N0. MB 16-24502.41. February 23, 2017

Cham, AL. 2016. Repeated Insult Patch Test (RIPT). Clinical research Laboratories, LLC Final Report. Study Number CRL2016-0601. October 12, 2016

Norman, K and Sheehan, D. 2015. Direct Peptide Reactivity Assay (DPRA). Institute for In Vitro Sciences, Inc.. Study Number: 15AF24.17500. August 2, 2015.

Norman, K and Willier E. 2015. Induction of Antioxidant-Response –Element Dependent Gene Activity and Cytotoxicity (Using MTT) in the Keratinocyte ARE-Reporter Cell Line Keratinosens. Institute for In Vitro Sciences, Inc. Study Number 15AF24.170001. October 23, 2015

Shelanski, HA and Shelanski MV. 1953. Proc. Sci. Sect. Toilet Goods Assoc. 19:46.

Appendix A – P-16-0510 Data Summaries
Prepared by [redacted]
4/24/17

The following studies were reviewed based on consideration of the Adverse Outcome Pathway (AOP) for dermal sensitization. The OECD Test Guidelines for these studies describe the AOP: The molecular initiating event is the covalent binding of electrophilic substances to nucleophilic centers in skin proteins (evaluated by the Direct Peptide Reactivity Assay, DPRA). The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as gene expression associated with specific cell signaling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways (evaluated by the KeratinoSens™ assay). The third key event is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, chemokines and cytokines (evaluated by the Human Cell Line Activation Test, h-CLAT). The fourth key event is T-cell proliferation, which is indirectly assessed in the murine Local Lymph Node Assay.

Direct Peptide Reactivity Assay (DPRA)
Norman, K and Sheehan, D. 2015. Direct Peptide Reactivity Assay (DPRA). Study Number: 15AF24.17500. Institute for In Vitro Science. August 2, 2015.

According to the OECD Test Guideline (442C), the DPRA is designed to address the protein reactivity molecular initiating event of the skin sensitization AOP. Specifically, the assay quantifies the reactivity of test chemicals towards model synthetic peptides containing either lysine or cysteine. Reactivity is based on the depletion of cysteine and lysine in the peptide. Custom synthetic peptides of lysine and cysteine (containing phenylalanine to aid in detection) were used in this assay. The purity of each peptide was 90-95%. A set of serially diluted peptide standards were prepared for each peptide; six standards were prepared at concentrations of 0.534 - 0.0167 mM. A seventh standard was prepared containing only dilution buffer. The PMN substance and positive control used in this assay (cinnamaldehyde) were both prepared at a concentration of 100 mM.

The final dosing solutions were prepared in triplicate for the PMN substance, positive control, and reference control in the pre-labeled autosampler vials.

Relative peptide concentration is measured by high performance liquid chromatography (HPLC) with gradient elution and ultraviolet (UV) detection at 220 nm.

The percent depletion was calculated for the PMN substance sample and the positive control (cinnamaldehyde) samples as shown below.

% Peptide Depletion = [1- PMN substance or Positive Control Peptide Peak Area / Mean Peptide Peak Area of Reference Control C] x 100.

The results indicate the average % peptide depletion was minimal, and EPA concluded **the results of the study indicate negative prediction for skin sensitization.**

KeratinoSens™ assay
Norman, K and Willier E. 2015. Induction of Antioxidant-Response –Element Dependent Gene Activity and Cytotoxicity (Using MTT) in the Keratinocyte ARE-Reporter Cell Line Keratinosens. Institute for In Vitro Science, Inc. Study Number 15AF24.170001. October 23, 2015

According to the OECD Test Guideline (442D), the KeratinoSens™ assay is designed to address the second key event in the AOP. Specifically, skin sensitizers may induce genes that are regulated by the antioxidant response element (ARE). The ARE-Nrf2 luciferase test method makes use of an immortalized adherent cell line. The cell line contains the luciferase gene under the transcriptional control of a constitutive promoter fused with an ARE element from a gene that is known to be up-regulated by contact sensitizers. The luciferase signal reflects the activation by sensitizers of endogenous Nrf2 dependent genes.

In this study, three definitive assays were run to determine the maximal induction (I_{max}), the concentration for maximal gene induction (CI_{max}), the EC1.5 value (concentration for a statistically significant induction of 50% above the solvent controls), and a mean IC_{50} (concentration leading to 50% viability relative to solvent controls) of the PMN substance. Cytotoxicity was evaluated using MTT.

Test concentrations of the PMN substance were 0.977, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500, 1000 and 2000 µM. The positive control was cinnamic aldehyde.
The test was evaluated in two trials. In the first trial, the positive control assay was determined to be invalid, so the assay was repeated. After 48 hours of incubation, gene induction was measured based on the luminescence associated with luciferase.

Positive results were based on the following criteria: 1) EC1.5 values below 1000 µM in at least 2 of 3 repetitions; 2) cellular viability >70% at the lowest concentration with a gene induction above 1.5, and 3) an apparent overall dose response which was similar between repetitions.

The results did not meet the criteria for positive results. EPA concluded **the results of the study indicate negative prediction for skin sensitization.**

Human Cell Line Activation Test (h-CLAT)
Carathers, MR. Human Cell Line Activation Test (h-CLAT). MB Research Labs. Research Project N0. MB 16-24502.41. February 23, 2017

According to the OECD Test Guideline (442E), the h-CLAT method addresses the third key event of the skin sensitization AOP. The method quantifies sensitizer-induced changes in the expression of cell surface markers associated with the process of activation of monocytes and dendritic cells in the human monocytic leukemia cell line THP-1. In this study, five controls were used:

- Positive control - 1-chloro-2,4-dinitrobenzene (DNCB, 4 µg/ml in dimethyl sulfoxide [DMSO]);
- Positive control - nickel sulfate (NiSO4, 100 µg/ml in 0.9%sodium chloride [saline]);
- Negative control - lactic acid (LA, 1000 µg/ml in saline);
- Vehicle control for DNCB - DMSO (0.2% in RPMI-10 medium); and
- Cell media control - RPMI-10 medium alone (100%)

Per the study report: "The assay was first conducted using only the controls, not the PMN substance, to check the reactivity of the cells. THP-1 human monocytic cells were seeded in 24-well plates at a concentration of approximately 1 x 10^6 cells in 0.5 ml of cell culture medium. Cells were dosed at one well per control and incubated for approximately 24 hours. The cells were then treated with propidium iodide (PI) plus antibody stain (for CD86 or CD54) to determine viability and induction of sensitization. Two independent viability screens were then conducted using the PMN substance, but not controls." In the PMN substance viability screens, the seeding density was approximately 1 x 10^6 cells in 0.5 ml of culture medium. Dosing was one well per concentration of the PMN substance/eight concentrations were tested. The incubation was for 24 hours followed by staining with PI. None of the concentrations tested produced a cell viability of less than 97.1%.

Four independent main tests were conducted. For each treatment, flow cytometry analysis was used to measure cell viability and the Mean Fluorescence Intensity (MFI) of the viable cells for CD86 and CD54 expression.

CD86 and CD54 expression was evaluated based on the Relative Fluorescence Intensity (RFI) using the following equation:

RFI =
MFI of chemical-treated cells **–** MFI of chemical-treated isotype cells /
MFI of solvent-treated cells **–** MFI of solvent-treated isotype cells

The OECD TG stipulates that an h-CLAT prediction is considered POSITIVE if at least one of the following conditions is met in 2 of 2 or in at least 2 of 3 independent runs, otherwise the h-CLAT prediction is considered NEGATIVE (Figure 1):

- The RFI of CD86 is equal to or greater than 150% at any tested concentration (with cell viability ≥ 50%);
- The RFI of CD54 is equal to or greater than 200% at any tested concentration (with cell viability ≥ 50%).

Treatment with the PMN substance in Main Test 1 produced positive responses for both CD86 and CD54, but the calculated RFI values were inconsistent and not dose-dependent.

Main Test 2 was invalid due to failure to pass the quality control acceptance criteria.

Since the results of the third main test were not consistent with those of the first main test, a fourth main test was conducted.

Main Tests 3 and 4 were valid tests, and the results were negative (e.g., RFI for CD86<150% and for CD54<200%).

Therefore, EPA concluded **the results of the study indicate negative prediction for skin sensitization.**

Human Repeated Insult Patch Test (HRIPT)
Cham, AL. 2016. Repeated Insult Patch Test (RIPT). Clinical research Laboratories, LLC
Final Report. Study Number CRL2016-0601. October 12, 2016

This study was based on a modified Shelanski – Shelanski human patch test method[1]. In this study, one hundred thirteen volunteers participated in evaluating the potential of the PMN substance (and another chemical) to elicit dermal irritation and/or induce sensitization. For the Induction Period, 0.15 ml of the test material was applied to a 3.63 cm^2 under occlusive patches applied to the upper back of each subject. The contact period was 24 hours. Patches were applied to the same site on Monday, Wednesday, and Friday for a total of 9 applications during the Induction Period.

The sites were graded by a CRL technician for dermal irritation 24 h after patch removal on Tuesday and Thursday, but 48 hours after patch removal on Saturday.

Challenge patches were applied to untreated test sites on the back, 10 to 21 days after the Induction Phase. After 24 hours, the patches were removed by a CRL technician and the test sites were evaluated for dermal reactions. The test sites were re-evaluated at 48 and 72 hours after application.

Of the one hundred twelve subjects that completed the study, the results for dermal irritation or sensitization were negative under the following test conditions: 0.15 ml of the test material applied to a 3.63 cm^2 occlusive patch. EPA concluded **the results of the study indicate negative prediction for skin sensitization.**

[1] H.A. Shelanski and M.V. Shelanski, Proc. Sci. Sect. Toilet Goods Assoc. 19:46, 1953.